

An *in Vivo* Replication-important Function in the Second Coding Exon of Tat Is Constrained against Mutation despite Cytotoxic T Lymphocyte Selection*

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Human and simian immunodeficiency virus (HIV/SIV) Tat proteins are specified by two coding exons. Tat functions in the transcription of primate lentiviruses. A plethora of *in vitro* data currently suggests that the second coding exon of Tat is largely devoid of function. However, whether the second exon of Tat contributes functionally to viral pathogenesis *in vivo* remains unknown. To address this question directly, we compared infection of rhesus macaques with an SIV, engineered to express only the first coding exon of Tat (SIVtat1ex), to counterpart infection with wild-type SIVmac239 virus, which expresses the full 2-exon Tat. This comparison showed that the second coding exon of Tat contributes to chronic SIV replication *in vivo*. Interestingly, in macaques, we observed a cytotoxic T lymphocytes (CTL) response to the second coding exon of Tat, which appears to durably control SIV replication. When SIV mutated in an attempt to escape this second Tat-exon-CTL, the resulting virus was less replicatively fit and failed to populate the host *in vivo*. Our study provides the first evidence that the second coding exon in Tat embodies an important function for *in vivo* replication. We suggest the second coding exon of Tat as an example of a functionally constrained “epitope” whose elicited CTL response cannot be escaped by virus mutation without producing a virus that replicates poorly *in vivo*.

atic phase of infection (7–9). Relevant to this concept, the selection of viral mutations associated with loss of CTL response has been found in HIV-1 as well as simian immunodeficiency virus (SIV) infections (10–12). Hence, in HIV-infected patients, CTL escape mutations in *nef*, *env*, and *gag* have been detailed (10). Indeed, escape from immunodominant CTL response is associated with progression to AIDS (13).

Current thinking suggests that vaccines, which induce CTL responses to HIV, although incapable of preventing infection, could provide partial or complete protection from AIDS progression. The efficacy of such CTL-inducing vaccines is, however, limited by the propensity to select for escape mutations (14). The extreme mutability of HIV as evidenced by its rapid development of resistance to reverse transcriptase and protease inhibitors is well documented (reviewed in Refs. 15 and 16). Hence, elucidation of immunologically important epitope(s) constrained against mutation by functional or structural value to viral survival would represent an important advance for vaccine development. Currently, no description of such a candidate exists.

Several HIV open reading frames such as *env* and *protease* are highly susceptible to second site compensatory mutations that rescue functions lost through original mutations (17–19). Functional constraints, which serve to limit mutation, would appear to be poorly operative for these viral proteins. On the other hand, more compact HIV open reading frames necessary for viral viability may be less tolerant of mutations. The HIV/SIV Tat protein is a small regulatory polypeptide whose function is essential for virus replication (reviewed in Ref. 20). There is evidence that CTL response against Tat may be more efficacious in controlling infection than comparable responses to viral structural proteins (21). However, the importance of such a finding is tempered by an observation that a CTL epitope in the first coding exon of SIV Tat quickly mutates upon acute immune selection (12). Recently, Walker and colleagues (22) have defined for HIV-1 an optimal epitope in the first coding exon of Tat and have suggested that CTL could be disease-effective if targeted to a region of Tat functionally constrained against immune-induced mutations. Here, we report that the second coding exon of Tat contains a function important for *in vivo* SIV replication in macaques and that CTL response against this region of Tat durably controls SIV replication. We provide evidence that CTL escape mutation in the second coding exon of Tat occurs with a prohibitive loss of

Human immunodeficiency virus is the causative agent of AIDS (1). HIV¹ infection causes AIDS after years of chronic, active infection. Viral replication, as measured by plasma viral genome copies, correlates well with disease progression (2–4). Increasing evidence suggests that cytotoxic T lymphocytes (CTL) play a critical role in the initial clearance of primary viremia (5, 6) as well as in the maintenance of the asymptom-

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¹ The abbreviations used are: HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; SHIV, simian-human immunodeficiency virus; CTL, cytotoxic T lymphocytes; CAT, chloramphenicol acetyltransferase; PBMC, peripheral blood mononuclear cells; TNF, tumor necrosis factor.

replicative fitness. Our results lead us to propose the second coding exon of Tat as an example of a functionally constrained epitope to which effective *in vivo* CTL response could control viral infection because CTL escape mutations in this epitope result in viruses that replicate poorly *in vivo*.

EXPERIMENTAL PROCEDURES

Animals—Protocols for the animals (adult rhesus macaques, 5–9 kg) were approved by the Tulane National Primate Research Center (TRPRC) institutional animal care and use committee. All animals were experimentally naive and were seronegative for D retrovirus and SIV.

Virus Clones—The proviral SIVmac239(open) clone, pNdbpuRQ, was generously provided by Riri Shibata. The SphI-MunI segment of pNdbpuRQ, was isolated, the MunI site was blunted with Klenow polymerase, and the segment was cloned into the SphI-SmaI sites of pSP72 (Promega, Madison, WI) to form the shuttle vector, pSP72SIV. The three point mutations, as described under "Results," were then introduced by PCR cloning, and the segment containing the mutations was cloned back into pNdbpuRQ to make pSIVtat1ex. For purposes of this study, SIV generated from the pNdbpuRQ clones is SIVmac239.

Viral Stocks—The viral stocks were prepared by lipofection of the plasmid DNAs into 293T cells (AIDS Reagent Program, National Institutes of Health, Bethesda, MD) with LipofectAMINE (Invitrogen). The cells were then cultured for 3 days. The supernatant was harvested, clarified, and aliquoted. Stock aliquots only underwent one freeze-thaw cycle. The TCID₅₀ of each stock was determined on CEM-X-174 cells (AIDS Repository) and calculated using the methods of Reed and Muench (23).

Rev Assay—Using LipofectAMINE (as above), 293T cells were transfected with 4 µg of pDM128-CAT (Rev-dependent CAT expression vector) (24) and with 2 µg of pSIVtat1ex, pSIVtat2ex, pUC, or pRSV-Rev (Rev expression vector) in 6-well plates. Cell lysates were prepared 48 h after transfection and analyzed for CAT content by CAT enzyme-linked immunosorbent assay (Roche Applied Science) according to the manufacturer's protocol.

Virus Infection—Each stock was diluted in serum-free RPMI medium to a concentration of 100 infectious units/ml on the day of infection. Each animal then received 1 ml of the appropriate stock through intravenous injection.

Branched DNA—Branched bDNA assays were performed at Bayer Corp. (Emeryville, CA) as described previously (25). Plasma from heparinized blood was separated and stored at -70 °C until it was assayed. The lower limit of detection was 1500 SIVmac viral genome copies/ml. The assay has a specificity of 96% (26).

PCR Cloning—After virus from plasma was pelleted, RNA was extracted with standard techniques. Reverse transcription was performed with primer, Out2 (see below), and enzyme, M-MuLV (New England Biolabs, Beverly, MA). Nested PCR was performed on the reverse-transcribed DNA with an outer primer pair (Out1, 5'-TGGAGGTTCT-GGAAGAAGT-3', and Out2, 5'-CTCCAAGCTGGTACACCAT-3') and an inner primer pair (In1, 5'-GGAGGTTCTGGAAGAAGTGA-3', and In2, 5'-GGATGGCGATAAGCAGCTGAT-3') from the *tat* region of SIVmac. PCR products were cloned using the Original TA cloning kit (Invitrogen), according to the manufacturer's protocol.

CD4+ Cell Staining and Quantification—T-cell numbers were evaluated by flow cytometry using fluorochrome-labeled monoclonal antibodies with the following specificities: CD3-fluorescein (clone SP34; Pharmingen), CD4-phycoerythrin (clone Leu3a; Immunocytometry Systems), and CD8-Per-CP (clone Leu2a; BD Biosciences). The relative numbers of CD3+CD4+ cells, CD3+CD8+ cells, and fluorescent beads, as determined by flow cytometry (FACSCalibur, BD Biosciences), were used to calculate the numbers of these cells per µl of blood according to the formula provided by the manufacturer's instructions.

γ-Interferon ELISPOT and Intracellular Cytokine Staining—Macaque PBMCs, purified by standard Ficoll separation and frozen in Me₂SO-freezing media, were thawed and examined for γ-interferon production after stimulation with SIV peptides. Nitrocellulose, microtiter plates (Millipore, Bedford, MA) were incubated with an anti-γ-interferon antibody (B27; Pharmingen) at 2 µg/ml overnight at 4 °C. After the wells were washed and blocked, PBMCs (1–4 × 10⁵) were added to each well in 100 µl of RPMI/10% fetal bovine serum. Peptide pools (2–3 peptides/pool) were added in 100 µl of RPMI/10% fetal bovine serum to a final concentration of 2–4 µg/ml. The cells and peptides were incubated at 37 °C, 5% CO₂ for 36 h. After washing, a second anti-γ-interferon antibody (Clone 7-86-1; Diapharma, West Chester, OH) at 1 µg/ml in 100 µl was added, and the wells were incubated for 1 h at 37 °C. Wells were washed again. Avidin-horseradish peroxidase (Vector

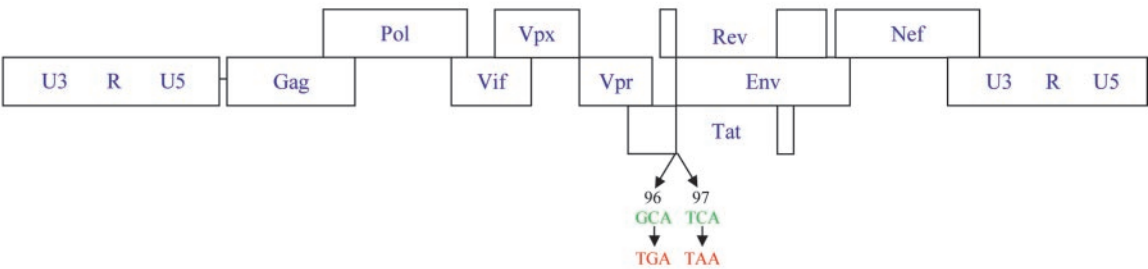
Laboratories, Burlingame, CA) was added in 100 µl, and the plate was incubated at 37 °C for 30 min. Wells were washed six times, and the spots were developed with undiluted 2,4-diaminobutyric acid (ResGen, Huntsville, AL) for ~10 min at room temperature. After the wells were rinsed with H₂O, the spots on the membrane of each well were counted under a stereomicroscope. All cell-peptide pool samples were run in duplicate. The Tat (15-mer) peptides were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health. The number of spot-forming cells/10⁶ PBMCs was calculated by subtracting the background (the number of spots in the control wells) and adjusting for cell number per well and then normalized by a factor of 6 to control for the freeze-thaw effect. In control studies, the freeze-thaw cycle reduced ELISPOT spot-forming cells by an average of 6-fold. All reported data were at least twice the level of the negative control. For intracellular cytokine staining, fresh PBMCs were separated from the whole blood of animal L840 drawn 3.5 years after infection. The final nine, 15-mer peptides (National Institutes of Health AIDS Research and Reference Reagent Program catalog numbers 5429–5437) of Tat were used to individually stimulate CD8+ T-cells. The peptides overlap by 11 amino acids and have at least 1 amino acid in the second exon of Tat. Staphylococcal enterotoxin B was used a positive control for activation. Cell staining, flow cytometric analysis, and data processing were done strictly according to the BD Biosciences protocol for rhesus blood. Antibodies used for the cell staining were a-CD3 fluorescein isothiocyanate (clone SP34, Pharmingen), α-CD8 PerCP (clone SK-1, Immunocytometry Systems), α-CD69 PE (clone L78, Immunocytometry Systems), and a-TNFα APC (clone 6401.1111, Immunocytometry Systems). For each peptide or staphylococcal enterotoxin B stimulation, the percentage of TNFα producing CD8- and CD69-positive lymphocytes was calculated by computer analysis. The results were analyzed using the BD Bioscience method.

RESULTS

The Second Coding Exon of Tat Is Important for SIV Replication in Macaque—Previously, we and others have shown that the second coding exon of Tat is important for *in vitro* HIV-1 replication in human PBMCs (27). To investigate this requirement *in vivo*, we constructed two otherwise isogenic forms of SIVmac239 differing only in their second coding exon of Tat. SIVtat1ex, which encodes only the first coding exon of tat, was constructed from SIVmac239 by mutating codons 96 and 97 of Tat from GCA-TCA to TGA-TAA (bases 6843–6847 in SIVmm239 GenBank™ M33262; underlined nucleotides were changed as indicated), which generated two consecutive stop codons at the end of the first coding exon of Tat (Fig. 1A). We confirmed by direct sequencing that pSIVtat1ex encodes only a 95-amino-acid truncated 1st-exon Tat. Because the *rev* reading frame (1+ relative to *tat*) overlaps *tat* in this region of the genome, we checked that our nucleotide changes were conservative for Rev function. Direct measurements using a Rev-dependent CAT reporter plasmid (24) verified that Rev function was unchanged between SIVtat1ex and SIVmac239 (Fig. 1B). We next generated viral stocks by transfecting SIVmac239 and SIVtat1ex in parallel into 293T cells. Three days after transfection, the tissue culture media were clarified and aliquoted as viral stocks. The p27 values of the viral stocks were 1.4 ng/ml for pSIVmac239 and 2.7 ng/ml for SIVtat1ex. TCID₅₀ values, as determined on CEM-X-174 cells, were 3083 and 4050 infectious units/ml for SIVmac239 and SIVtat1ex, respectively.

We infected intravenously six adult macaques with 100 TCID₅₀ of either SIVmac239 (two animals, M154 and N361) or SIVtat1ex (four animals, L840, L855, L882, and N200). Blood samples were collected serially for viral load and CD4 measurements. The viral load in all six animals peaked between days 14 and 17 (Fig. 2, A and B) after infection. The observed viral peaks were roughly equivalent (~10⁸ copies/ml) in the two groups and comparable with values documented previously in the majority of our animals infected with SIVmac (28). In the post-acute period (> 6 weeks), the two SIVmac239-infected animals had high set point plasma viral load (>10⁷ copies/ml). By comparison, the four SIVtat1ex-infected animals controlled

A)



B)

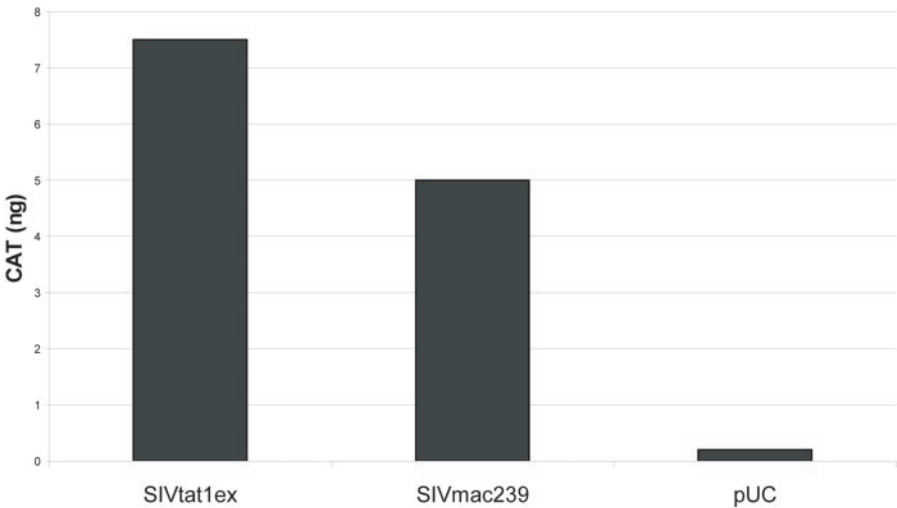


FIG. 1. Construction of a SIV molecular clone which expresses only the first exon of Tat. A, schematic representation of SIVmac239 and its SIVtat1ex derivative. SIVtat1ex differs from SIVmac239 only in that codons 96 and 97 (BCA TCA) from the latter were changed to TGA TAA in the former. B, Rev-dependent CAT expression. 293T cells were co-transfected with a Rev-dependent CAT expression vector, pDM128-CAT, separately with SIVtat1ex, SIVmac239, or control pUC18. Cell lysates were harvested and analyzed for CAT production using enzyme-linked immunosorbent assay. Replicate assays confirmed that there was no statistically significant difference in CAT activity with SIVtat1ex *versus* SIVmac239.

viremia relatively better. Two (N200 and L882) had viral loads consistently near 10^6 copies/ml, whereas the other two (L840, L855) had viral load values that became virtually undetectable by days 60–160 after infection.

A More Severe Disease Course Is Seen in SIVmac239-infected than SIVtat1ex-infected Macaques—Other than their *tat* gene, SIVmac239 and SIVtat1ex are isogenic. Hence, a comparison of the disease courses engendered by the two viruses should reveal the contribution (if any) of the second Tat exon to *in vivo* pathogenesis. We, thus, examined the CD4⁺ cell profile and the clinical outcomes in our six infected macaques.

In the two SIVmac239 animals (M154 and N361) CD4⁺ T-cell counts, after infection, declined rapidly and progressively, reaching a nadir of ~ 100 cells/ μ l (Fig. 2C). Both macaques developed pneumonia, anorexia, and weight loss at 4 and 7 months after infection, respectively, and had to be euthanized. By comparison, although CD4⁺ T-cell counts also fell initially, the four SIVtat1ex animals did much better. Two SIVtat1ex animals (N200 and L882) stabilized their CD4⁺ T-cell counts to between 250 and 500 cells/ μ l; the other two SIVtat1ex animals (L840 and L855), after an initial decline, raised their CD4 counts to approximately ≥ 1000 cells/ μ l by the fourth week of infection (Fig. 2D). The clinical courses were also significantly better in the SIVtat1ex group. Although L882 did have weight loss, thrombocytopenia, and anemia after 9

months and was euthanized at the 10th month after infection secondary to severe respiratory distress, the other three SIVtat1ex macaques did well. N200 had stable CD4⁺ T-cell count of ~ 500 cells/ μ l and remained healthy for more than 2 years after infection. L840 and L855 had long term control of viremia and stable CD4 counts for more than 1 year; both remained clinically well more than 3 years after infection. Thus, all four SIVtat1ex animals did better than their SIVmac239 counterparts, and three of the four SIVtat1ex macaques survived well beyond the demise of both SIVmac239 animals.

Rapid Change of 1-Exon Tat to 2-Exon Tat Prognosticates a Poor Clinical Outcome—413 days into our study, we observed a sudden rise in viral load in animal L855 (Fig. 3A), which was accompanied by a precipitous drop in CD4 cell count (Fig. 3B). This contrasted with a largely unchanged viral load and stable CD4 cell count in the companion L840 animal. We were puzzled by the sudden divergence in the presentations of L882 and L855 and sought to understand whether the virus in one of these two animals might have changed to the more pathogenic two exon Tat-encoding SIVmac239.

To address the question of virus reversion from SIVtat1ex to SIVmac239, we carefully examined plasma viral RNAs collected serially from all four SIVtat1ex animals, N200, L855, L882, and L840 (Table I). *tat* sequence in the viral RNAs was

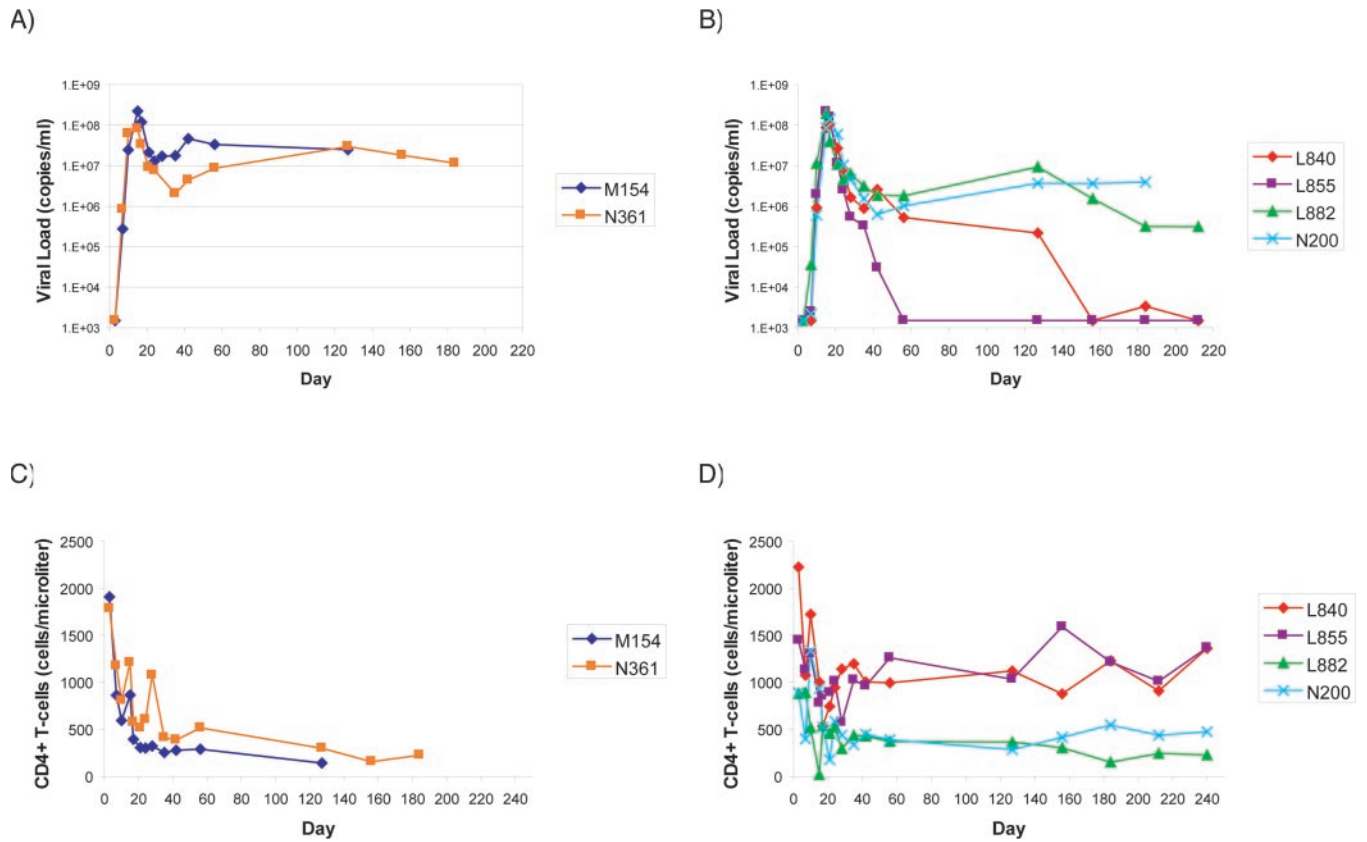


FIG. 2. Plasma viral loads and CD4+ T-cell counts in the SIVmac239 (A and C; animals M154, N361)- and SIVtat1ex (B and D; animals L840, L855, L882, N200)-infected macaques. The viral load or SIV RNA copies per ml of plasma, as measured by a branched DNA assay, are plotted *versus* day after infection. Peripheral blood CD4+ T-cell counts were determined by flow cytometry at indicated times after infection. The counts, expressed in CD4+ T-cells per ml of whole blood, are also plotted *versus* day after infection.

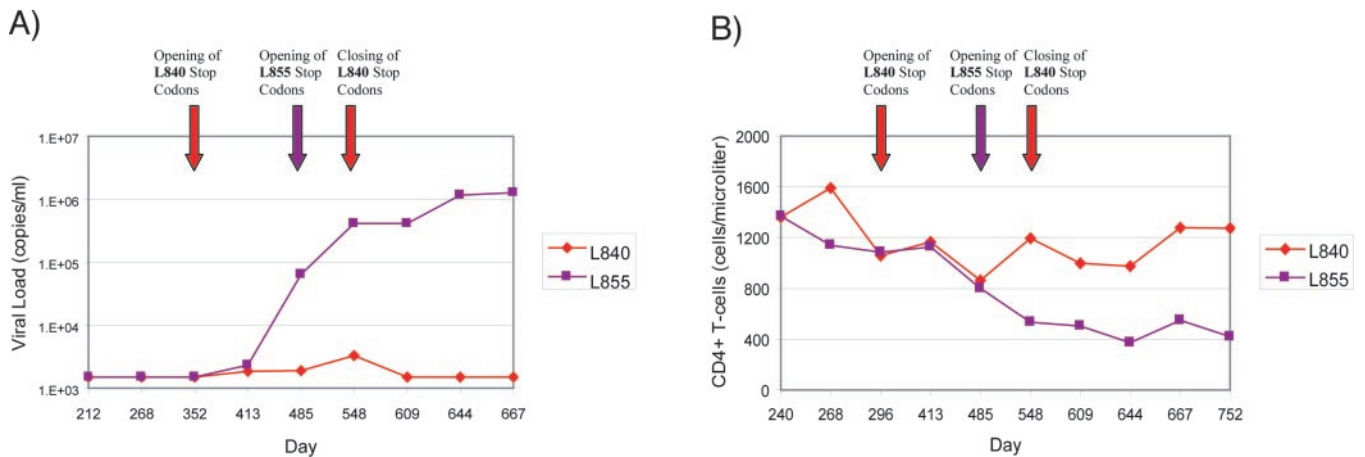


FIG. 3. Comparison of SIVtat1ex-infected macaques, the viral loads of L840 and L855 (A), and CD4+ T-cell counts (B) at the indicated times after infection. The arrows (light red for L840 and dark red for L855) denote the time of opening or closing of the *tat* stop codons.

amplified by reverse transcriptase-PCR, cloned into plasmid vectors, and sequenced. Proviral DNA was sequenced only a single time (*i.e.* the final L840 time point; Table I) when viral RNA inexplicably could not be amplified by reverse transcriptase-PCR. For each time point, the status of the *tat* stop codons was determined from several independent clones. (Note: the denominator in Table I indicates the number of clones sequenced; the numerator represents the number of clones with the indicated sequence at codons 96 and 97. SIVtat1ex commenced with the TGA TAA sequence at positions 96 and 97).

In all SIVtat1ex animals, stop codons at positions 96 and 97 were maintained through the first 4 weeks of infection. Inter-

estingly, as early as day 56, one SIVtat1ex animal, L882, had reverted both stop codons, opening them in eight of eight sequenced clones. This stop-codon-opening was *bona fide* reversion and not sample contamination since the reverted amino acids at 96 and 97, Ser-Ser, were different from the wild-type amino acids, Ala-Ser. Indeed, this rapid change from SIVtat1ex to SIVmac239 in L882 apparently explains its SIVmac239-like (*i.e.* M154- and N361-like) demise at the 10th month after infection. Hence, a revised interpretation of the comparative courses in the six animals is that three out of three original/rapidly reverted SIVmac239 animals (*i.e.* M154, N361, L882) uniformly succumbed to disease/death by the 10th month of infection, whereas three out of three SIVtat1ex animals (*i.e.*

TABLE I
Tat codons 96 and 97 in SIVtat1ex infected animal over time

Day	N200	L855	L882	L840
35		TGA TAA 11/11		
42	TGA TAA 6/6	TGA TAA 4/7 TGA TAG 3/7	TGA TAA 19/24 TCA TAA 5/24	TGA TAG 3/5 TGA TAA 2/5
56	TGA TAA 7/7		TCA TCA 8/8	
127	TGA TAG 7/7	TGA TAG 5/5	TCA TCA 4/5 TCA TTA 1/5	TGA TAG 6/11 TCA AA 5/11
156	TGA TAG 5/5		TCA TCA 7/12 TGA TAG 4/12 TCA TAA 1/12	
184	TGA TAG 5/5	TCA TGA 7/15 TGA TAG 8/15		TCA TAA 10/14 TGA TAA 2/14 TGA TCA 1/14 TCA TCA 1/14
212			TCA TCA 4/4	
268	TGA TAG 4/4		TCA TCA 7/8 TCA TCG 1/8	TCA TCA 7/8 TCA TAA 1/8
352		TGA TAG 10/10		
413	TGA TAG 10/10	TCA CGA 8/12 TCA CAA 1/12 TGA TAG 3/12		TCA TCA 7/10 TGA TCA 1/10 TGA TAG 1/10
485	TGA TAG 5/5			TGA TAG 4/5 TCA TCA 1/1
609		TCA CGA 8/10 TCA CCA 1/10 TGA TAG 1/10		
667				TGA TAG 10/10
773				TGA TAG 1/1
955	TGA TCA 5/6 TGA TAA 1/6	TCA CCA 12/12		
1386				TGA TAG 7/10 TCA TCA 3/10

N200, L855, L840) remained clinically well more than 2 years after infection.

Other than L882, the three remaining SIVtat1ex animals, N200, L855, L840, maintained firmly both Tat stop codons through the first year of infection. Nevertheless, a detailed perusal of their sequence results was informative. For example, the opening of the stop codons in L855 (*i.e.* 9 out of 12 clones opened) by day 413 temporally correlated with its rise in viral load and concomitant decline in CD4+ cell count (Fig. 3; Table I). When considered with the outcomes observed for M154 and N361 (SIVmac239 animals) and L882 (a rapid revertant to SIVmac239), the L855 finding supports that a change from one-exon Tat- to two-exon Tat-encoded virus is likely causative *in vivo* of increased viral load and decreased CD4 cell counts.

A second set of instructive findings was seen in the SIVtat1ex animal L840. Early after infection, L840 had low viral loads and high CD4+ cell counts (Fig. 3). Interestingly, when sequence data were examined, by day 268 after infection, L840 presented evidence for attempted reversions to SIVmac239, opening both stop codons in seven out of eight sequenced clones (Table I). Curiously, unlike L882, the *in vivo* attempt of L840 to mutate from SIVtat1ex to SIVmac239 failed to succeed durably. Indeed, when day 485 viral RNAs were sequenced, L840 again showed an *in vivo* dominant SIVtat1ex virus (*i.e.* four out of five clones had the TGA-TAG sequence; Table I). We note that unlike the finding for L885, where mutation of SIVtat1ex virus to SIVmac239 occurred in the face of rising viral load and falling CD4 counts, the re-emergence of SIVtat1ex virus in L840 occurred in a setting of low viral load and high CD4+ cell counts (Fig. 3). One interpretation is that the L840 immune system succeeded (where L855 failed) in selecting against the more potently replicating SIVmac239 virus, permitting the re-emergence of the less potent, but immune unselected, SIVtat1ex virus. Indeed, to date, after more than 1500 cumulative days of infection, L840 remains clinically healthy with a normal CD4+ T-cell count and a low viral load (~2000 copies/ml). Taken together, our collective data are fully compatible with the notion that SIV that expresses the 2-exon form of Tat is better than its 1-exon counterpart in *in vivo* replication/pathogenesis.

Cellular Immune Response Directed to the Second Coding Exon of Tat Is Found in L840—The different outcomes in L882, L855, and L840 to nascent reversions of SIVtat1ex to SIVmac239 prompted us to consider whether differential immune responses (*i.e.* CTL) to the second coding exon of Tat might

explain the varying courses. We assessed CTL activity using a γ -interferon ELISPOT assay (29). In this assay, PBMCs, which had been cryopreserved, were stimulated with two pools of peptides from the second coding exon of Tat. The number of cells, which were stimulated by peptides to produce γ -interferon, was counted based on *in situ* immunological detection of spots of cells affixed to membrane. We were limited in this analysis by the fact that we did not successfully anticipate the serial cryopreservation of PBMC samples from all animals. Hence, a complete comparison of CTL responses could not be performed. Nevertheless, in limited side-by-side comparisons of successfully preserved PBMCs from M154, N361, L855, L882, L840, and N200 at ~6 months after infection, we found that L882 had the worst, whereas L840 had the best, cellular responses to two different pools of Tat second coding exon peptides (Fig. 4A). The results are compatible with the idea that an effective CTL directed to the second coding exon of Tat in L840 selected against the nascent transition (day 268; Table I) of SIVtat1ex to the better replicating SIVmac239, permitting the re-emergence by day 485 (Table I) of SIVtat1ex (which is not selected against by CTLs specific for the second coding exon of Tat). The less robust CTL responses in L882 and L855 were apparently insufficient to control SIVmac239, hence allowing for nascent forms of the better replicating SIVmac239 to quickly dominate *in vivo*.

The cellular response of L840 to the second exon of SIVmac239 Tat was further independently verified using intracellular cytokine staining. Intracellular cytokine staining was performed after stimulation of L840 PBMCs with peptides from the second exon of Tat. The cells were stained with an anti-TNF α antibody and then analyzed by flow cytometry (Fig. 4B). The results suggest that L840 has CTL activity against two epitopes in the second exon of Tat, one within peptides 2 and 3 and the other within peptides 7 and 8. Since these peptides overlap, these data suggest that the epitopes recognized by L840 correspond to amino acids 92–104 and 112–124 of SIVmac239 Tat.

We also compared anti-SIV humoral responses in our infected macaques to see whether differences in this immune parameter might explain the various viral courses. By immunoblotting assays, we found that SIV-specific humoral immune responses in L840, L855, and N200 were similar (Fig. 5) and could unlikely account for the different viral courses observed in these animals. The other three macaques, M154, N361, and L882, had little to no humoral immunity against SIV; this is an

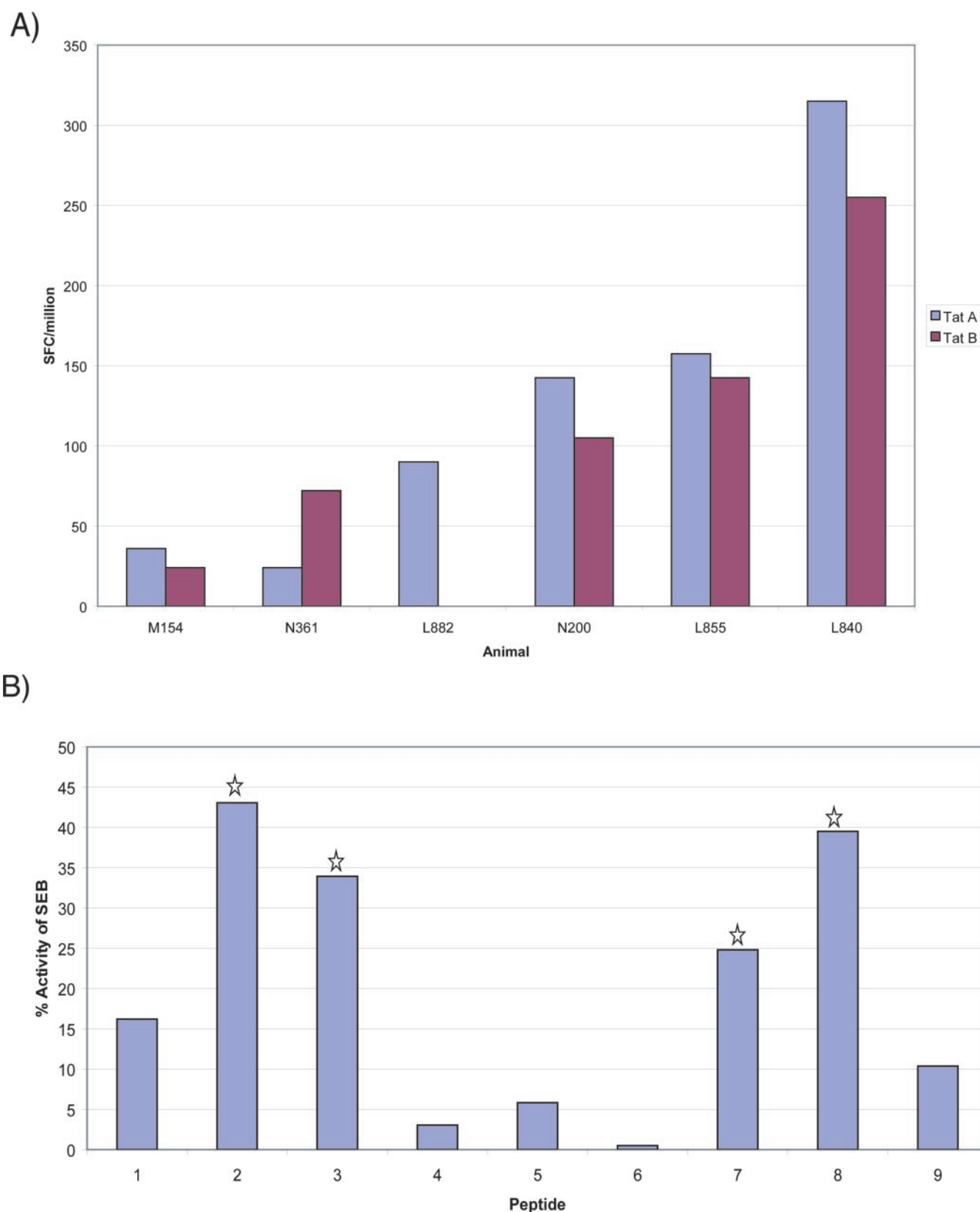


FIG. 4. *In vivo* CTL assays. A, ELISPOT assay with two pools of SIV Tat 2nd-exon peptides. PBMCs were from the indicated animals at ~6 months after infection. Spot-forming cells (SFC)/ 10^6 PBMCs are shown for each animal. B, L840 PBMCs produced $\text{TNF}\alpha$ in response to Tat exon 2 peptide stimulation. PBMCs from animal L840 produce $\text{TNF}\alpha$ in response to stimulation with peptides from exon 2 of SIVmac239 Tat. The data are shown as the percentage of activity relative to that of staphylococcal enterotoxin B (SEB) stimulation after subtraction of the background level. Peptides that elicited responses statistically above background ($p < 0.05$) are indicated with a star.

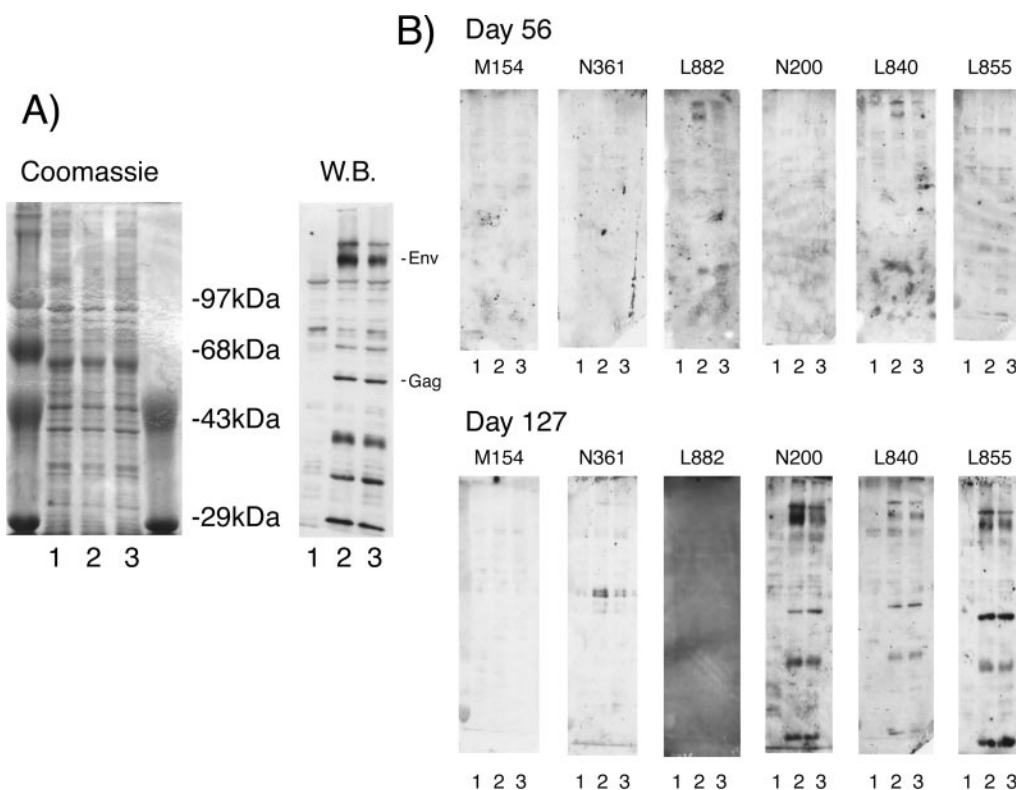


FIG. 5. **Humoral immune response to SIV in infected macaques.** A, control Western blotting of SIVMac239 proteins using hyperimmune simian serum. HeLa cells mock-transfected (lane 1) or transfected with SIVMac239 molecular clone (loaded in duplicates in lanes 2 and 3) were resolved in 10% SDS-polyacrylamide gel. Two identical gels were loaded, and after electrophoresis, one was stained with Coomassie Blue (left), and the second was transferred to Immobilon-P membrane (W.B.) and then probed with hyperimmune serum to SIVMac239 (gift from Vanessa Hirsch). Envelope (Env) and Gag (Gag) proteins are indicated. B, Western blotting of polyvinylidene difluoride strips identical to that shown in panel A with 1:500 dilution of sera from M154, N361, L882, N200, L840, and L855 on day 56 (top) and day 127 (bottom) after infection.

oft-observed characteristic of rapid progressors in this model of SIV infection (30).

Human Findings Support the Contributory Role of the Second Coding Exon of Tat for *in Vivo* Virus Replication—The above findings from SIVtatlex-infected macaques may or may not be generalizable to HIV-1 infection in humans. For purposes of comparison, it is, of course, not possible to deliberately inoculate an engineered HIVtatlex into human. However, it occurred to us that such an inoculation may already have been inadvertently performed in three human individuals. Three laboratory workers (LWF, LWR, LWS) were accidentally infected in 1985 and 1990 with laboratory-passaged HIV-1 IIIB. Although unrecognized at the time, recent analyses (31) show that IIIB, unlike primary HIV-1 isolates, has a premature stop codon in its *tat* gene, rendering it a virtue HIVtatlex equivalent for SIVtatlex. By retrospectively studying the viral courses in LWF, LWR, and LWS, we could ask two questions. The first question is whether the opening of 1-exon-HIV Tat to 2-exon-HIV Tat also correlated with virus replication and disease progression in human. The second question is whether there is evidence to support the notion that immune selection against the second exon of Tat can drive a better replicating HIV-2-exon-Tat *in vivo* to its less potent HIV-1-exon-Tat form.

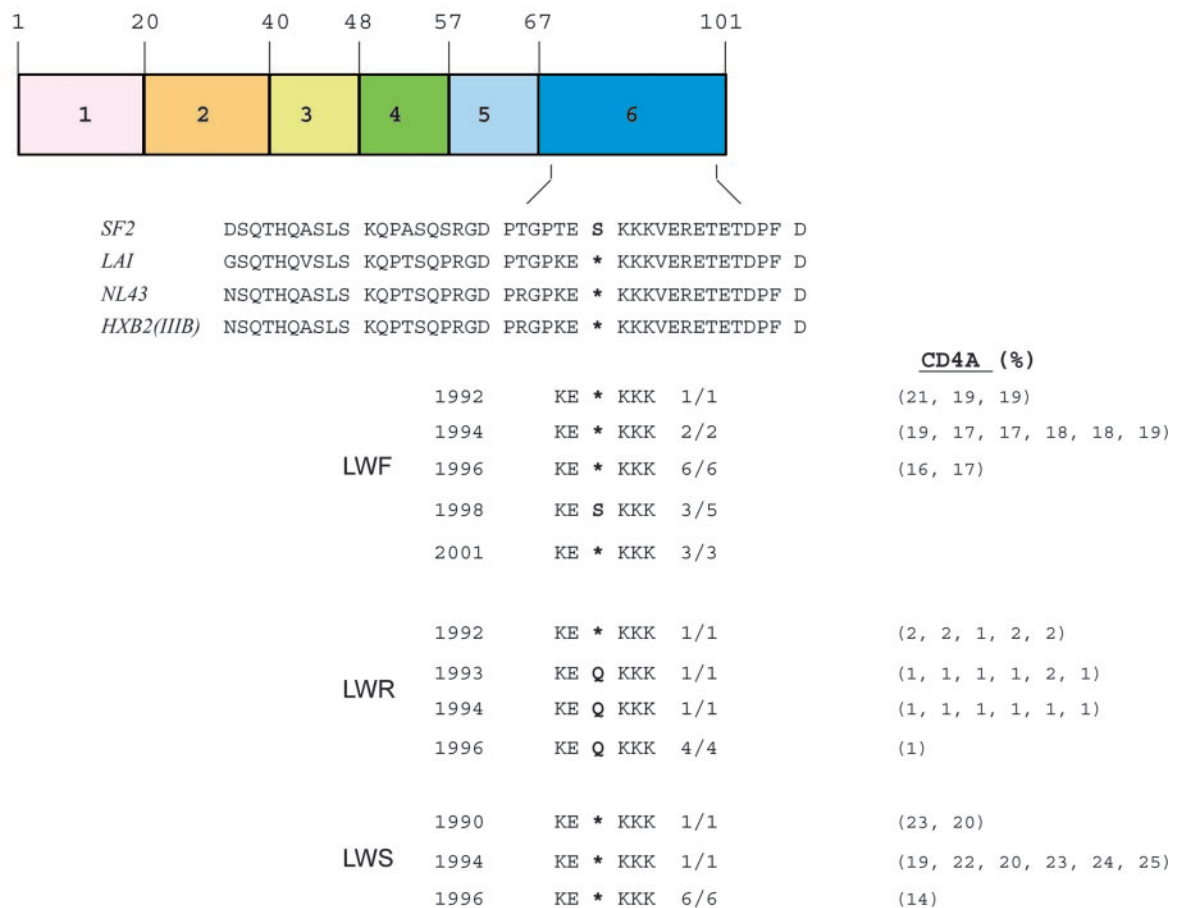
We analyzed serial HIV sequences in LWF, LWR, and LWS focusing on the premature stop codon of IIIB in *tat*. In our small sampling set, the HIV IIIB findings largely agreed with the results from our SIV-infected macaques. For instance, patient LWR, like macaque L882, had a rapidly demonstrable and stably maintained change from his/her 1-exon Tat IIIB-virus to a new 2-exon Tat HIV. Remarkably, this change in LWR, which parallels the change of SIVtatlex to SIVmac239 in L882, also

accompanied an L882-like precipitous decline in CD4 cell count (Fig. 6A, bottom right) with rapid progression within 1 year (32) to AIDS disease. Thus, the LWR scenario for HIV/human is essentially the same as the L882 scenario for SIV/macaque. Considered together, both findings support the notion that the second Tat exon of primate lentiviruses conserves a function generally important for HIV/SIV replication/pathogenesis *in vivo*.

We next asked whether human immune response to the second coding exon of Tat might also contribute to controlling *in vivo* HIV replication. Here, the course of patient LWF was instructive. LWF was infected in 1985 but then remained clinically well for the next 11 years (up through 1996) with robust CD4 counts of around $<500/\text{mm}^3$ (and CD4A of $\sim 16\text{--}19\%$). We noted with great interest that the IIIB Tat stop codon of LWF remained intact for 13 years until 1998 when, like macaque L840, mutation to a new 2-exon Tat IIIB virus was observed (in three out of five sequenced samples; Fig. 6A, bottom). Remarkably, when LWF sequences were re-examined 3 years later (*i.e.* in 2001), like the finding for macaque L840, the 2-exon-Tat IIIB form was undetectable; the original 1st-exon-Tat virus re-emerged as the sole form (in three out of three sequences; Fig. 6A, bottom). This, pending further verification, suggests that an *in vivo* immune response to the second coding exon of Tat eliminated the more potent 2-exon-Tat virus permitting the maintenance of the less replication competent 1-exon-Tat IIIB virus.

The final patient LWS remained clinically well through 1994 (information beyond 1994 was not available to us). Like macaque N200, consistent with his/her "benign" clinical course, LWS firmly maintained the IIIB virus in its 1-exon-Tat format (Fig. 6A, bottom). Overall, the collective data from six ma-

A)



B)

Tat CTL Epitope



FIG. 6. *In vivo* behavior of HIV-1 tat in infected individuals. A, reversion of stop codon in the second coding exon of HIV-1 Tat in three infected laboratory workers (LWF, LWR, and LWS). Top, schematic representation of the HIV-1 Tat domains modeled after that shown in Jeang *et al.* (31). The laboratory strains LAI, NL43, and HXB2 (IIIB) all contain a premature stop codon (*) in the second coding exon not found in wild primary isolates, which have a Tat sequence like that shown for SF2. Bottom, sequential evolution of the stop codon in LWF, LWR, and LWS with the number of sequences represented (numerator) and the number of individual clones sequenced (denominator) shown as a fraction at the right. CD4A% values (multiple sampled values within each indicated year) are shown at far right. B, location of the B58 CTL epitope in the 2nd exon of HIV-1 Tat. The schematic is adapted from data in the HIV Molecular Immunology Data base (<http://hiv-web.lanl.gov/content/immunology/maps/ctl/Tat.html>).

caques and three human are consistent with the presence of an *in vivo* replication/pathogenesis function in 2-exon-Tat, which is absent from 1-exon-Tat.

DISCUSSION

We have constructed an infectious SIVtat1ex virus that expresses a 1-exon tat gene. SIVtat1ex, when compared with an otherwise isogenic 2-exon tat SIVmac239, permitted us to ask two questions. First, is there a contribution by the second coding exon of Tat to *in vivo* virus replication/pathogenesis?

Second, if the second coding exon of Tat is functionally important, would its importance override the normal attempts by virus to mutate to escape *in vivo* selection by CTL? The latter question is particularly significant to developing a durable CTL-eliciting vaccine. Currently there is no example of a CTL response, which has not prompted successful mutational escape *in vivo* by HIV-1.

Our results support the novel conclusion that the second coding exon of Tat, thought previously to be devoid of function *in vitro*, is important for *in vivo* virus replication/pathogenesis.

In infected macaques, we observed that two SIVmac239 animals (M154 and N361), and one SIVtat1ex animal (L882) whose virus mutated quickly to SIVmac239, all had uniformly higher viral loads, lower CD4⁺ cell counts, and more rapid progression to simian AIDS (Fig. 1) than their three SIVtat1ex counterparts. "Non-reverted" SIVtat1ex animals (L840 and L855) remained clinically well for extended durations, showed good viremia control, and raised their CD4⁺ T-cell counts in the chronic periods after infection (Fig. 2). When the Tat stop codons did open in macaque L855 on day 413 (Table I), this opening correlated with increased viral load and decreased CD4⁺ T-cell count (Fig. 3), further reinforcing a link between the second Tat exon and *in vivo* SIV replication/pathogenesis. Likewise, in three human individuals infected with 1-exon Tat HIV, the opening of the HIV IIIB-Tat stop codon in patient LWR also correlated with the emergence of low CD4⁺ T-cell count and rapid progression to AIDS. Collectively, the findings implicate a contributory function in the second coding exon of Tat to *in vivo* replication and pathogenesis during the chronic phase of infection.

We do not currently understand what is the function encoded by the second exon of Tat. There are several possibilities. Full-length HIV-1 Tat has been shown to have several roles, including the modulation of host gene expression (33–37), activation of resting T-cells (38), induction of chemokine production (39), and direct suppression of antigen specific CD8⁺ T-cell immune response *in vivo* (40). The second coding exon of Tat has also been shown to have a propitious effect on HIV-1 replication in tissue culture (41, 42). Contribution to one or more of these effects by the second exon of Tat could explain its *in vivo* role for replication and pathogenicity.

Allen *et al.* (12) had reported previously that a strong CTL response was elicited in macaques to amino acids 28–35 in the first coding exon of SIV Tat. However, as discussed by those authors, because this portion of Tat has no *in vivo* function, viruses mutated in amino acids 28–35 (rendering the CTL response effete) quickly emerged and populated the infected host. Our finding that the second coding exon of Tat is functionally important for *in vivo* replication/pathogenesis raises the possibility that SIV may not be able to mutate and escape CTL directed to this segment and yet remain viable. Relevant to the consideration of this possibility, we found that our SIV-infected macaques did mount a CTL response to the second Tat exon and that the animal (L840) with the most robust Tat-CTL had a superior clinical course (Fig. 4).

Our SIVtat1ex virus responded to 2nd-exon Tat CTL differently from the way the SIV of Allen *et al.* (12) responded to 1st-exon Tat CTL. Whereas the virus of Allen *et al.* (12) virus quickly mutated its 1st-exon Tat and permanently escaped such CTL, our SIVtat1ex appeared unable to evade CTL raised to the 2nd-exon of Tat. This is illustrated by the behavior of the SIVtat1ex virus in macaque L840. Hence, on day 127 after infection, 100% of the virus of L840 (11/11; Table I) was in 1-exon Tat form (*i.e.* TGA, TAG, or TAA). However, 141 days later, by day 268, fully 87.5% of the virus of L840 had mutated to a 2-exon Tat form (seven out of eight clones had opened both stop codons; Table I). Subsequently, presumably, in response to the development of 2nd-Tat exon CTL in L840 (Fig. 4), by day 667, all traces of 2-exon Tat SIV was gone; 10 out of 10 sequenced clones showed the complete return of virus to the SIVtat1ex format (Table I). Curiously, after 100% of the L840 virus had "returned" to 1-exon Tat form by day 667 (therefore, in principle, fully escaped from CTL specific to the second exon of Tat), a second cycle of 1–2-exon evolution reinitiated. Thus, on day 1386, 30% of the virus of L840 again presented in 2-exon Tat form (3/10; Table I).

HIV and SIV predominantly escape CTL by single amino acid mutations within the targeted epitope (13, 43–48). Successful, long term suppression of viremia by CTL presumably requires immune targeting to epitope(s) in which any mutation is associated with a significant loss of viral function or fitness. In L840, SIV escaped CTL directed against the second exon of *tat* through epitope deletion (via truncation of the Tat reading frame), although truncation of Tat came with a significant loss of viral fitness. Similar epitope deletions have been described as a mode of escaping nef CTL *in vitro* and *in vivo* (47, 49). Based on our understanding of the lentivirus literature, what is strikingly unusual about the L840 scenario is its oscillation between 1-exon-Tat and 2-exon-Tat viruses? Although we do not fully understand the details that govern this oscillation, one notion, which we favor, is that maintaining the function of the 2nd-Tat exon must be important to the *in vivo* viability of the L840 virus. Thus, this functional value of the second exon of Tat to the virus appears to override the mutation of this epitope for purposes of escaping host immune selection. Hence, escaped virus (*i.e.* SIVtat1ex) returns to unescaped form (*i.e.* SIVmac239) because the escaped form (we reason) is much less viable and is disfavored *in vivo*. Possibly, this implies that SIV (and by extrapolation, HIV) may not be able to evade (via mutation) long term immune control by CTL specific to 2nd-exon-Tat. Interestingly, there is evidence for a CTL epitope in the second exon of HIV-1 Tat (Fig. 6B).

The macaque SIV model has been used extensively for vaccine development (50). Two groups have shown that immunization with Tat resulted in lower simian-human immunodeficiency virus (SHIV) viral loads (51–54). On the other hand, two other studies, one with SHIV and the other with SIVmac239 (the virus used in this study), showed no difference in viral load, despite strong cellular immune responses against Tat (55, 56). Based on our findings, it is likely that the quality of the cellular immune response to Tat may have dictated the different outcomes. Directing a CTL response to a functionless *versus* a function-containing Tat epitope, which mutates either at no or at significant replication cost to the virus, likely produces alternate outcomes. Our data on the 2nd-coding exon of Tat appear to provide the first inkling of a functionally constrained "immutable" epitope in primate lentivirus to which an effective virus-controlling CTL could be elicited such that the virus either fails to escape or escapes at a significant cost to its *in vivo* replicative fitness. If this interpretation is correct, then prechallenge immune response against the second exon of *tat* could be effective in controlling *in vivo* lentiviral replication. We propose that the second coding exon of *tat* be considered for testing as a vaccine in macaques, and pending that outcome, be considered further as a possible HIV-1 vaccine candidate.

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